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(57) Abstract

A method of increasing basal glucose transport, or increasing the level of the GLUT1 glucose transporter, in a cell or subject which includes administering an antagonist of the cellular protein the cellular protein Ubc9, to thereby increase basal glucose transport or increasing the level of GLUT1.

INDEPENDENT REGULATION OF BASAL AND INSULIN-STIMULATED GLUCOSE TRANSPORT

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The invention relates to methods and compounds for independently regulating basal and insulin-induced glucose uptake in cells, tissues, or organisms, e.g., by regulation of the activity of the GLUT1 and GLUT4 glucose transporter proteins.

10 **Background of the Invention**

The six-carbon monosaccharide glucose is a principal metabolic fuel of mammals and many lower organisms. In humans and other mammals, close regulation of the level of glucose in the blood and extracellular fluids is essential to assure glucose availability to tissues that require it as a source of metabolic

15 energy, and also to protect the organism from injurious effects of hyperglycemia. Glucose movement into cells is essential for its use in various cellular metabolic pathways. Regulated glucose uptake in certain cell types, including skeletal muscle and fat cells, plays a critical role in the maintenance of normal blood and tissue glucose concentrations. Glucose movement out of cells in tissues such as

20 liver and kidney may also have a significant role in maintaining normal blood and extracellular fluid glucose levels by preventing its decrease into the hypoglycemic range.

Summary of Invention

In general, the invention features, a method of increasing basal glucose

25 transport, or increasing the level of the GLUT1 glucose transporter, e.g., cell surface GLUT1, in a cell or subject comprising:

administering an antagonist of the cellular protein the cellular protein Ubc9,

thereby increasing basal glucose transport or increasing the level of GLUT1.

30 In preferred embodiments,, the increase of basal glucose transport, or of a level of GLUT1, e.g., cell surface GLUT1, is unaccompanied by an increase in insulin-stimulated glucose uptake.

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In preferred embodiments, the increase of basal glucose transport, or of a level of GLUT1, e.g., cell surface GLUT1, is unaccompanied by an increase of a level of the GLUT4 glucose transporter, e.g., cell surface GLUT4.

In preferred embodiments, the antagonist of UBC9 is a peptide or protein
5   molecule, e.g., an enzymatically inactive form of Ubc9, e.g., a GLUT4 or GLUT1 binding fragment of Ubc9 in which residue 93 of Ubc9 is other than cysteine, and is, e.g., alanine.

In preferred embodiments, the subject is a human or other animal in need of an increased level of basal glucose transport, e.g., a subject having a disorder
10   characterized by insufficient levels of basal glucose transport, having damaged neuronal tissue, e.g., tissue damaged by stroke; hyperglycemia; or an insulin resistant state, e.g., diabetes. In this and other methods described herein, an insufficient level of basal transport can be a normal physiological level, e.g., in situations where it is desirable to increase the level above normally observed
15   levels. The basal levels of glucose transport may not be less than normal in neuronal tissue, e.g., tissue damaged or at risk of damage by stroke, hyperglycemia, or diabetes, but increased levels of basal glucose transport will reduce or prevent damage to this tissue.

In preferred embodiments,, a cell is treated, and the cell is from such a
20   subject.

In another aspect, the invention features a method of increasing basal glucose transport, or increasing a level of GLUT1, e.g., cell surface GLUT1, in a cell or subject comprising:

inhibiting an interaction between Ubc9 and GLUT1,
25   thereby increasing basal glucose transport or increasing the level of GLUT1.

In preferred embodiments,, the increase of basal glucose transport, or of a level of GLUT1, e.g., cell surface GLUT1, is unaccompanied by an increase in insulin-stimulated glucose uptake.

In preferred embodiments,, the increase of basal glucose transport, or of a
30   level of GLUT1, e.g., cell surface GLUT1, is unaccompanied by an increase of a level of GLUT4, e.g., cell surface GLUT4.

In preferred embodiments,, the subject is a human or other animal in need of an increased level of basal glucose transport, e.g., a subject having a disorder

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characterized by insufficient levels of basal glucose transport, having damaged neuronal tissue, e.g., tissue damaged by stroke, hyperglycemia, or an insulin resistant state, e.g., diabetes. The basal levels of glucose transport may not be less than normal in neuronal tissue, e.g., tissue damaged or at risk of damage by

5   stroke, hyperglycemia, or diabetes, but increased levels of basal glucose transport will reduce or prevent prevent damage to this tissue. In preferred embodiments, a cell is treated, and the cell is from such a subject. (In this and other methods herein, an insufficient level of basal transport can be a normal physiological level, e.g., in situations where it is desirable to increase the level above normally

10   observed levels.)

In preferred embodiments,, the subject is a human or other animal having a disorder characterized by high levels of basal glucose transport, e.g., hyperglycemia or unwanted cell proliferation, e.g., benign or malignant tumors, e.g., an islet cell tumor, or a tumor which produces insulin or an insulin-like

15   factor, and in general cancer.

In other preferred embodiments, a cell is treated, and the cell is from such a subject.

In another aspect, the invention features a method of inhibiting the attachment of sentrin to GLUT1 or GLUT4. The method includes: inhibiting an

20   interaction between Ubc9 and GLUT1, thereby inhibiting the attachment of sentrin to GLUT1 or GLUT4.

In preferred embodiments,, the subject is a human or other animal having a disorder characterized by high levels of basal glucose transport, e.g., hyperglycemia or unwanted cell proliferation, e.g., benign or malignant tumors,

25   e.g., an islet cell tumor, or a tumor which produces insulin or an insulin-like factor, and in general cancer.

In other preferred embodiments, a cell is treated, and the cell is from such a subject.

In a preferred administering an antagonist of the cellular protein the

30   cellular protein Ubc9 is administered to inhibit attachement of sentrin.

In preferred embodiments, the antagonist of UBC9 is a peptide or protein molecule, e.g., an enzymatically inactive form of Ubc9, e.g., a GLUT4 or GLUT1

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binding fragment of Ubc9 in which residue 93 of Ubc9 is other than cysteine, and
is, e.g., alanine.

In preferred embodiments, a cell is treated.

In another aspect, the invention features, a method of increasing basal
5   glucose transport , or increasing a level of the GLUT1, e.g., the level of cell
surface GLUT1, in a cell or subject. The method includes:

inhibiting an interaction between Ubc9 and GLUT4,
thereby increasing basal glucose transport or increasing the level of GLUT1.

In preferred embodiments,, the increase of basal glucose transport, or of a
10   level of GLUT1, e.g., cell surface GLUT1, is unaccompanied by an increase in
insulin-stimulated glucose uptake.

In preferred embodiments,, the increase of basal glucose transport, or of a
level of GLUT1, e.g., cell surface GLUT1, is unaccompanied by an increase of a
level of GLUT4, e.g., cell surface GLUT4.

15   In preferred embodiments,, the subject is a human or other animal in need
of an increased level of basal glucose transport, e.g., a subject having a disorder
characterized by insufficient levels of basal glucose transport, e.g., having
damaged neuronal tissue, e.g., tissue damaged by stroke; hyperglycemia; or an
insulin resistant state, e.g., diabetes. (In this and other methods herein, an
20   insufficient level of basal transport can be a normal physiological level, e.g., in
situations where it is desirable to increase the level above normally observed
levels.) (In this and other methods herein, an insufficient level of basal transport
can be a normal physiological level, e.g., in situations where it is desirable to
increase the level above normally observed levels.) The basal levels of glucose
25   transport may not be less than normal in meuronal tissue, e.g., tissue damaged or
at risk of damage by stroke, hyperglycemia, or diabetes, but increased levels of
basal glucose transport will reduce or prevent prevent damage to this tissue.

In preferred embodiments, a cell is treated, and the cell is from such a
patient.

30   In another aspect, the invention features a method of decreasing basal
glucose transport, or decreasing a level of GLUT1, e.g., cell surface GLUT1, in a
cell or subject. The method includes: promoting an interaction of Ubc9 or
sentrin or a function analog of sentrin moiety with GLUT4 and/or GLUT1,

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thereby decreasing the basal glucose transport and/or decreasing the level of
GLUT1.

In preferred embodiments, the decrease of basal glucose transport, or of a
level of GLUT1, e.g., cell surface GLUT1, is unaccompanied by a decrease in
insulin-stimulated glucose uptake.

In preferred embodiments,, the decrease of basal glucose transport, or of a
level of GLUT1, e.g., cell surface GLUT1, is unaccompanied by a decrease of a
level of GLUT4, e.g., cell surface GLUT4.

In preferred embodiments, the method includes administering a Ubc9
moiety, or an inducer of UBC9 cellular content or function.

In preferred embodiments, the subject is a human or other animal in need
of decreased levels of basal glucose transport, e.g., a subject having a disorder
characterized by high levels of basal glucose transport, e.g., hyperglycemia or
unwanted cell proliferation, e.g., benign or malignant tumors, e.g., an islet cell
tumor, or a tumor which produces insulin or an insulin-like factor, and in general
cancer. The level of basal glucose transport may be normal, but a decrease in
basal glucose transport, e.g., in neuronal, retinal, renal, vascular, or other tissues
damaged as a consequence of hyperglycemia in diabetes mellitus, may reduce
damage to such tissue.

In other preferred embodiments, a cell is treated, and the cell is from such
a subject.

In a preferred embodiment UBC that is associated with GLUT4
and/orGLUT is activated.

In another aspect, the invention features a method of decreasing basal
glucose transport, or decreasing a level of GLUT1, e.g., cell surface GLUT1, in a
cell or subject. The method includes:

attaching sentrin (or functional analog of sentrin) to GLUT4 and/or
GLUT1, thereby decreasing the basal glucose transport, or decreasing the level of
GLUT1.

In preferred embodiments,, the decrease of basal glucose transport, or of a
level of GLUT1, e.g., cell surface GLUT1, is unaccompanied by a decrease in
insulin-stimulated glucose uptake.

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In preferred embodiments,, the decrease of basal glucose transport, or of a level of GLUT1, e.g., cell surface GLUT1, is unaccompanied by a decrease of a level of GLUT4, e.g., cell surface GLUT4.

In preferred embodiments,, the subject is a human or other animal in need of decreased levels of basal glucose transport, e.g., a subject having a disorder characterized by high levels of basal glucose transport, e.g., hyperglycemia or unwanted cell proliferation, e.g., benign or malignant tumors, e.g., an islet cell tumor, or a tumor which produces insulin or an insulin-like factor, and in general cancer. The level of basal glucose transport may be normal, but a decrease in basal glucose transport, e.g., in neuronal, retinal, renal, vascular, or other tissues damaged as a consequence of hyper glycemia indiabetes mellatus, may reduce damage to such tissue.

In other preferred embodiments, a cell is treated, and the cell is from such a subject.

In preferred embodiments,, the method includes administering a Ubc9 moiety or sentrin or a function analog of sentrin or an inducer of UBC9 cellular content or function.

In another aspect, the invention features a method of decreasing insulin-stimulated glucose transport, or decreasing the level of GLUT4, e.g., cell surface GLUT4, in a cell or subject comprising: administering an antagonist of Ubc9 thereby decreasing insulin-stimulated glucose transport or decreasing the level of GLUT4.

In preferred embodiments,, the decrease of insulin-stimulated transport, or of a level of GLUT4, e.g., cell surface GLUT4, is unaccompanied by a decrease in basal glucose uptake.

In preferred embodiments,, the decrease of insulin-stimulated transport, or of a level of GLUT4, e.g., cell surface GLUT4, is unaccompanied by a decrease in a level of GLUT1, e.g., cell surface GLUT1.

In preferred embodiments,, the antagonist of UBC9 is a peptide or protein molecule, e.g., an enzymatically inactive form of Ubc9, e.g., a GLUT4 or GLUT1 binding fragment of Ubc9 in which residue 93 of Ubc9 is other than cysteine, and is, e.g., is alanine.

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In preferred embodiments,, the subject is a human or other animal having a need of decreased levels of basal glucose transport, e.g., a subject having a disorder characterized by high levels of insulin-stimulated transport, e.g., hyperglycemia, e.g., certain benign or malignant tumors, e.g., islet cell tumors

5   producing insulin, or tumors producing other insulin like factors, or inoperable cancers.

In another aspect, the invention features a method of decreasing insulin-stimulated glucose transport, or decreasing a level of GLUT4, e.g., cell surface GLUT4, in a cell or subject comprising: inhibiting an interaction between Ubc9

10 and GLUT1,
thereby decreasing insulin-stimulated glucose transport or decreasing the level of GLUT4.

In preferred embodiments, the decrease of insulin-stimulated transport, or of a level of GLUT4, e.g., cell surface GLUT4, is unaccompanied by a decrease

15 in basal glucose uptake.

In preferred embodiments, the decrease of insulin-stimulated transport, or of a level of GLUT4, e.g., cell surface GLUT4, is unaccompanied by a decrease in a level of GLUT1, e.g., cell surface GLUT1.

In another aspect, the invention features a method inhibiting interaction

20 between UBC and GLUT4, in a cell or subject comprising:
administering a compound, e.g., a coupound described herein, which binds to UBC or GLUT4, and thereby inhibits the interaction.

In preferred embodiments, an antagonist of UBC9, e.g., is a peptide or protein molecule, e.g., an enzymatically inactive form of Ubc9, e.g., a GLUT4 or

25 GLUT1 binding fragment of Ubc9 in which residue 93 of Ubc9 is other than cysteine, and is, e.g., is alanine, is administered.

In another aspect, the invention features a method of increasing insulin-stimulated glucose transport or increasing the level of GLUT4, e.g., cell surface GLUT4 in a cell or subject. The method includes:

30   promoting an interaction of a Ubc9 moiety or sentrin or a function analog of sentrin with GLUT4 and/or GLUT1, thereby increasing insulin-stimulated glucose transport or increasing the level of GLUT4.

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In preferred embodiments, the increase of insulin-stimulated transport, or of a level of GLUT4, e.g., cell surface GLUT4, is unaccompanied by an increase in basal glucose uptake.

In preferred embodiments, the increase of insulin-stimulated transport, or
5   of a level of GLUT4, e.g., cell surface GLUT4, is unaccompanied by an increase in a level of GLUT1, e.g., cell surface GLUT1.

In preferred embodiments,, the subject is a human or other animal in need of an increased level of basal glucose transport, e.g., a subject having a disorder characterized by insufficient levels of insulin-stimulated glucose transport, e.g.
10   damaged neuronal tissue, e.g. tissue damaged by stroke, hyperglycemia, or diabetes or other insulin resistant states. The basal levels of glucose transport may not be less than normal in neuronal tissue, e.g., tissue damaged or at risk of damage by stroke, hyperglycemia, or diabetes, but increased levels of basal glucose transport will reduce or prevent damage of this tissue.

15   In preferred embodiments, a cell is treated and the cell is from such a subject.

In preferred embodiments, the method includes administering a Ubc9 moiety, or an inducer of the UBC9 cellular content or function.

In another aspect, the invention features a method of increasing insulin-
20   stimulated glucose transport or increasing the level of GLUT4, e.g., surface GLUT4 in a cell or subject. The method includes:

attaching sentrin (or functional analogs of sentrin) to GLUT4 and/or GLUT1,

thereby increasing insulin-stimulated glucose transport or increasing the level of
25   GLUT4.

In preferred embodiments, the increase of insulin-stimulated transport, or of a level of GLUT4, e.g., cell surface GLUT4, is unaccompanied by an increase in basal glucose uptake.

In preferred embodiments, the increase of insulin-stimulated transport, or
30   of a level of GLUT4, e.g., cell surface GLUT4, is unaccompanied by an increase in a level of GLUT1, e.g., cell surface GLUT1.

In preferred embodiments, the subject is a human or other animal in need of an increased level of basal glucose transport, e.g., a subject having a disorder.

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Characterized by insufficient levels of insulin-stimulated glucose transport, damaged neuronal tissue, e.g. tissue damaged by stroke; hyperglycemia; or an insulin resistant state, e.g., diabetes.

In preferred embodiments, the method includes administering a Ubc9 moiety, or an inducer of UBC9 cellular content or function.

In another aspect, the invention features a method of evaluating a compound, e.g., for the ability to modulate, for example to inhibit or promote, an interaction between Ubc9 and GLUT4 including:

a) providing a GLUT4 moiety;

b) contacting the GLUT4 moiety with said compound; and

c) evaluating the ability of the GLUT4 moiety to interact with, e.g., bind, said compound, the ability of the compound to interact with the GLUT4 moiety being indicative of the ability to modulate the interaction between GLUT4 and Ubc9, thereby evaluating a compound for the ability to modulate the interaction between Ubc9 and GLUT4.

In another aspect, the invention features a method of evaluating a compound, e.g., for the ability to modulate, for example to inhibit, an interaction between Ubc9 and GLUT1 including:

a) providing a GLUT1 moiety;

b) contacting the GLUT1 moiety with said compound; and

c) evaluating the ability of the GLUT1 moiety to interact with, e.g., bind, said compound, the ability of the said compound to interact to the GLUT1 moiety GLUT1 being indicative of the ability to modulate the interaction between GLUT1 and Ubc9, thereby evaluating a compound for the ability to modulate the interaction between Ubc9 and GLUT1.

In another aspect, the invention features a method of evaluating a compound, e.g., for the ability to modulate, e.g. inhibit, an interaction between GLUT4 and Ubc9. The method includes:

a) forming a reaction mixture which includes a Ubc9 moiety, a GLUT4 moiety and the compound; and

b) determining if the compound modulates an interaction between the GLUT4 moiety and the Ubc9 moiety.

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In another aspect, the invention features the a method of evaluating a compound, for the ability to modulate, e.g. inhibit, an interaction between GLUT1 and Ubc9. The method includes:

a) forming a reaction mixture which includes a Ubc9 moiety, a GLUT1
5   moiety and the compound; and

b) determining if the compound modulates an interaction between the GLUT1 moiety and the Ubc9 moiety.

In another aspect, the invention features a fragment of GLUT4 which interacts with Ubc9, and which preferably includes the first eleven amino acids of
10   the C-terminal domain of GLUT4.

In another aspect, the invention features a fragment of GLUT1 which interacts with Ubc9, and which preferably includes the first eleven amino acids of the C-terminal domain of GLUT1.

In another aspect, the invention features a fragment of Ubc9 capable of
15   interacting with GLUT4, e.g., the first eleven amino acids of the C-terminal domain of GLUT4.

In another aspect, the invention features a fragment of Ubc9 capable of interacting with GLUT1, e.g., the first eleven amino acids of the C-terminal domain of GLUT1.

20   In another aspect, the invention features a purified preparation of a GLUT4 or GLUT1 binding fragment of Ubc9 which includes residue 93 of Ubc9.

In another aspect, the invention features a purified preparation of a GLUT4 or GLUT1 binding fragment of Ubc9 in which residue 93 of Ubc9 is other than cysteine.

25   In another aspect, the invention features a purified preparation of a GLUT4 or GLUT1 binding fragment of Ubc9 in which residue 93 of Ubc9 is alanine.

In another aspect, the invention features a method of treating a subject having a disorder characterized by unwanted basal levels (GLUT1-mediated
30   levels) of a glucose transport. The method includes: promoting the attachment of sentrin (or functional analog of sentrin) to GLUT1, thereby decreasing basal glucose transport.

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In preferred embodiment attachment of sentrin (or functional analog of sentrin) is promoted by providing a Ubc9 moiety to the subject.

In preferred embodiments, the disorder is: disorders characterized by unwanted cell proliferation, e.g., benign or malignant tumors, e.g., an islet cell tumor, or a tumor which produces insulin or an insulin-like factor, and in general cancer; hyperglycemia.

In preferred embodiments, the disorder is neuronal damage, e.g., stroke damage; hyperglycemia; or an insulin resistant state, e.g., diabetes.

In another aspect, the invention features a method of treating a subject having a disorder characterized by unwanted basal levels (GLUT1-mediated levels) of glucose transport. The method includes: promoting the attachment of sentrin to GLUT1, thereby decreasing basal glucose transport.

In preferred embodiment attachment of sentrin is promoted by providing a Ubc9 moiety to the subject.

In preferred embodiments, the disorder is: disorders characterized by unwanted cell proliferation, e.g., benign or malignant tumors, e.g., an islet cell tumor, or a tumor which produces insulin or an insulin-like factor, and in general cancer; hyperglycemia.

In another aspect, the invention features a method of treating a subject having a disorder characterized by unwanted basal levels (GLUT1-mediated levels) of a glucose transport. The method includes:

promoting the attachment of sentrin to GLUT1, thereby decreasing basal glucose transport.

In preferred embodiment attachment of sentrin is promoted by providing a Ubc9 moiety to the subject.

In preferred embodiments, the disorder is: disorders characterized by unwanted cell proliferation, e.g., cancer; hyperglycemia.

In preferred embodiments, the disorder is neuronal damage, e.g., stroke damage; hyperglycemia; or an insulin resistant state, e.g., diabetes.

In another aspect, the invention features a method of evaluating a compound, for e.g., for the ability to treat a disorder, e.g. an insulin or glucose related disorder, e.g., diabetes, where there is an unwantedly high level of blood glucose. (An unwantedly low level can be a physiologically normal level in a

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situation where it is desirable to raise the level above normal levels.) The method includes:

      a) providing a GLUT4 moiety;

      b) providing a Ubc9 moiety; and

5   c) forming a reaction mixture containing the GLUT4 moiety, the Ubc9 moiety, and other components required for the attachment of sentrin to GLUT4 and the

      compound; thereby, evaluating the ability of the compound to modulate, e.g. promote, the attachment of sentrin to GLUT4.

10   In another aspect, the invention features a method of evaluating a compound for, e.g., the ability to treat a disorder, e.g. an insulin or glucose related disorder, e.g., diabetes, where there is an unwantedly high level of blood glucose. The method includes:

      a) providing a GLUT1 moiety;

15   b) providing an antagonist of Ubc9; and

      c) forming a reaction mixture containing the GLUT1 moiety, the Ubc9 antagonist, and other components required for the inhibition of the attachment of sentrin to GLUT1 and the compound; thereby, evaluating the ability of the compound to modulate, e.g. inhibit, the attachment of sentrin to GLUT1.

20   In another aspect, the invention features a method of evaluating a compound for, e.g., the ability to treat a disorder, e.g. an insulin or glucose related disorder where there is an unwantedly low level of blood glucose. The method includes:

      a) providing a GLUT1 moiety;

25   b) providing a Ubc9 moiety; and

      c) forming a reaction mixture containing the GLUT1 moiety, the Ubc9 moiety, and other components required for the attachment of sentrin to GLUT1 and the

      compound; thereby, evaluating the ability of the compound to modulate,

30   e.g. promote, the attachment of sentrin to GLUT1.

      In another aspect, the invention features a method of evaluating a compound,e.g. for the ability to treat a disorder, e.g. an insulin or glucose related

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disorder, where there is an unwantedly low level of blood glucose. The method includes:

a) providing a GLUT4 moiety;

b) providing an antagonist of Ubc9; and

5   c) forming a reaction mixture containing the GLUT4 moiety, the Ubc9 antagonist,   and other components required for the inhibition of the attachment of sentrin to GLUT4 and the compound;

thereby, evaluating the ability of the compound to modulate, e.g. inhibit, the attachment of sentrin to GLUT4.

10   The invention allows treatment of blood glucose abnormalities in diabetes and other disease states by independently modulating insulin-dependent (GLUT4 dependent) and insulin-independent (GLUT1 dependent) glucose transport. Insulin effects on glucose uptake in states of insulin resistance or deficiency can be modulated by specifically modifying insulin-dependent glucose transport.

15 Insulin-dependent glucose transport can be inhibited in states of excess insulin or decreased insulin counterregulatory hormone action. By specifically increasing insulin-independent glucose transport, blood glucose levels can be decreased in the absence of insulin, providing an alternative to insulin for the treatment of diabetes. By inhibiting insulin-independent glucose transport, hypoglycemia can

20 be corrected or prevented, e.g., during periods of low nutrient intake, when basal glucose transport in addition to even a low level of insulin-stimulated glucose transport can be excessive.

Ubc9 has been found to bind to the GLUT4 and GLUT1 glucose transporters. Through its catalytic activity, Ubc9 promotes the attachment of

25 sentrin, a small protein, to the glucose transporters. Ubc9 binds to a specific region of the glucose transporters, which includes the first 11 amino acids of the C-terminal domain that extends into the cellular interior after the last transmembrane segment (equivalent to residues 467-477 in GLUT4). A synthetic peptide corresponding to this 11 amino acid sequence can inhibit the binding of

30 Ubc9 to the glucose transporters.

The introduction of a normal version of Ubc9 into cells results in decreased basal glucose transport and augmented insulin-dependent glucose transport. However, the introduction of a catalytically inactive mutant form of

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Ubc9 into cells results in increased basal glucose transport and decreased insulin-
dependent glucose transport. The effect of increased cellular content of the
normal Ubc9 enzyme is associated with a marked increase in the cellular content
of the GLUT4 glucose transporter and a decrease in GLUT1, whereas the inactive
5   mutant Ubc9 increases GLUT1 transporter content. Thus, the Ubc9 enzyme has
opposite effects on GLUT4 and GLUT1 cellular content and opposite effects on
basal and insulin-stimulated glucose transport. The discovery of the Ubc9
binding to the glucose transporters, the demonstration of its opposite effects on
basal and insult-stimulated glucose transport, and the demonstration of its
10   opposite effects on the cellular abundance of GLUT4 and GLUT1 provides a
novel system for investigating and modulating glucose transporter function.
       As used herein, the term "antagonist" refers to a substance which reduces
the level of Ubc9 activity, e.g. by binding competitively or by binding
noncompetitively with the Ubc9 ligand.
15   As used herein, the term "subject" refers to an organism, for example a
human, a monkey, a pig, a horse, a cow, or a rodent, e.g. a rat or a mouse. A
subject can be an animal which is a model for an insulin or glucose related
disorder, e.g., a NOD mouse.
       As used herein, the term "interaction" includes binding. In the case of
20   UBC9, it can include the attachment of sentrin.
       As used herein, the term "GLUT4 moiety" refers to GLUT4, or an analog
or fragment thereof which interacts with Ubc9, e.g., a moiety which serves as a
substrate for Ubc9 mediated sentrin attachment.
       As used herein, the term "GLUT1 moiety" refers to GLUT1 or an analog
25   or fragment thereof which interacts with Ubc9, e.g., a moiety which serves as a
substrate for Ubc9 mediated sentrin attachment.
       As used herein, the term "Ubc9 moiety" refers to Ubc9 or an analog or
fragment thereof which interacts with either GLUT1 or GLUT4.
       Other embodiments are discussed herein.
30   **Figures**
       Figure 1 is a bar graph illustrating the effects of increased cellular content
of Ubc9 on glucose uptake in L6 myoblasts.
       Detailed Description of Invention

15.

Alterations in glucose transporter function contribute significantly to many disease states. Increased transporter function can lead to low glucose levels (hypoglycemia). This can result in symptoms ranging from change of mood, loss of motor function, and cognitive deficiencies, to loss of consciousness, coma, and

5   death. Causes of increased transporter function and hypoglycemia include excess insulin dosage in diabetes mellitus (insulin reactions), excess insulin production by tumors, decreased insulin counterregulatory hormones as a consequence of certain endocrine deficiency states, and elevated levels of certain cytokines, such as tumor necrosis factor-α, in bacterial sepsis and other forms of serious illness.

10   Decreased transporter function can lead to elevated glucose levels (hyperglycemia). This can result in symptoms ranging from weakness and confusion, to coma and death. Causes of decreased transporter function and hyperglycemia include decreased insulin and/or insulin resistance in diabetes mellitus, and increased insulin counterregulatory hormones leading to insulin

15   resistance in states of hormone excess caused by primary endocrine disease or by the physiological stress response to severe illnesses.
     GLUT4

The movement of glucose across mammalian cell membranes is mediated by transmembrane proteins designated glucose transporters. As a charged

20   molecule, glucose cannot move freely across the lipid environment of cellular membranes. Glucose transporter proteins are thought to be positioned in the surface membranes of cells, such that they extend back-and-forth through the membrane a total of 12 times and thus form a structure that can somehow bring the glucose molecule through the membrane. Multiple glucose transporter

25   isoforms have been described, which differ in their tissue distribution, their regulation by hormones, muscle exercise, and other factors, and their affinity for glucose and other monosaccharides. One of these glucose transporter isoforms, designated GLUT4, is particularly abundant in skeletal muscle, cardiac muscle, and adipose tissue. The extent of glucose transport in these tissues is markedly

30   stimulated by the hormone insulin, and there is evidence that this occurs both by promoting the movement GLUT4 glucose transporters from inside the cell to the cell surface membranes, and by increasing the intrinsic activity of the GLUT4 transporters. These effects of insulin on the GLUT4 glucose transporter are an

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important part of the mechanism that maintains normal blood glucose levels. In
transgenic mice, in which a gene expressing increased levels of the GLUT4
glucose transporter has been introduced, the resulting elevated levels of GLUT4
have been shown to augment insulin sensitivity and to result in lower blood
5   glucose levels. By contrast, when GLUT4 glucose transporter levels are
decreased in mice by removing the normal GLUT4 gene, the animals develop
hyperglycemia and diabetes. In addition to its importance in mediating insulin
effects on glucose uptake, function of the GLUT4 glucose transporter in muscle is
stimulated by exercise. This has important effects on blood glucose levels and
10   the provision of glucose as a metabolic fuel to support exercise.
     GLUT1
          GLUT1 has a much wider tissue distribution and less functional
dependence on insulin than GLUT4. The GLUT1 transporter is present in
skeletal muscle, cardiac muscle, and adipose tissue, but at lower levels than
15   GLUT4. In these tissues, the GLUT1 transporter is of particular importance in
mediating basal glucose uptake. Basal glucose uptake is the uptake of glucose at
low insulin levels or in the absence of insulin. Additional glucose transporter
isoforms have been described with different patterns of tissue expression
thanGLUT4 or GLUT1.
20   The GLUT4 transporter is distributed predominantly at intracellular sites
in the absence of insulin and undergoes marked movement to the surface
membrane of cells in the presence of insulin. Thus, a cell with mostly GLUT4
transporters will have low basal glucose uptake and this will increase
dramatically with insulin. Skeletal muscle and adipose tissue have predominantly
25   GLUT4 transporters and they exhibit this pattern of low basal and highly insulin-
regulated glucose uptake. By contrast, the GLUT1 transporter distributes to a
much greater extent into the surface membranes of cells in the absence of insulin,
a smaller percentage is localized to intracellular sites, and insulin has a smaller
effect on GLUT1 movement. A cell with predominantly GLUT1 transporters,
30   such as fetal cells or brain cells, will have a high rate of glucose uptake in the

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absence of insulin, and insulin will have relatively little stimulatory effect on glucose uptake.

These properties of GLUT4 and GLUT1 fit well with what is observed herein in the L6 myoblasts (skeletal muscle cell line from newborn rats), when

5   the cellular content of Ubc9 was changed. Increased Ubc9 leads to increased GLUT4 and decreased GLUT1, and this is associated with a decrease in basal glucose uptake and an increase in the magnitude of insulin stimulation of glucose uptake. Decreased Ubc9 (achieved by introducing the inactive mutant form of Ubc9) leads to an increase in GLUT1 and no change in GLUT4, since it already

10   is very low in the L6 myoblasts. This is associated with an expected increase in basal glucose uptake and a decrease in the magnitude of insulin stimulation of glucose uptake.

While not wishing to be bound by theory, the following mechanistic observations can be made. Several possible mechanisms discussed herein may

15   act independently or cooperatively. Ubc9 binding to GLUT4 transporters may stabilize them, decrease their rate of breakdown, increase their half-life (normally about 7 hours), and thus lead to an increased amount of GLUT4 in the cell. This could result directly from Ubc9 binding to the transporter, or from Ubc9-catalyzed coupling of sentrin to the transporter (sentrinization) and an effect of

20   sentrin on GLUT4 turnover. Such a stabilization of the GLUT4 transporter could result from a direct inhibitory effect of the Ubc9 or sentrin on the normal mechanism that breaks down transporters. There could be a more indirect effect of Ubc9 or sentrin, for example, altering cellular localization of the GLUT4 transporter so that it is spatially isolated from the protein degradation system.

25   Ubc9 and/or sentrin may have opposite effects on the GLUT1 transporter, i.e. increasing its breakdown and shortening its half-life through the same types of direct or indirect mechanisms. The attachment of ubiquitin to proteins can affect

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both their localization in cells and their degradation. Since Ubc9 is homologous
to ubiquitin-conjugating enzymes, and sentrin is homologous to ubiquitin, it is
reasonable to consider either or both of these mechanisms as the basis for sentrin
effects on glucose transporters.

5   The opposite effects of Ubc9 on GLUT4 and GLUT1 transporters may be
explained by opposite direct effects of Ubc9/sentrin on these two transporter
isoforms. It is also possible that Ubc9 has its predominant effects on only one of
these isoforms, and that the changes in the other are secondary. For example,
Ubc9/sentrin could stabilize GLUT4 and lead to its increased content in cells. As

10   GLUT4 increases, this could activate a Ubc9-unrelated mechanism for controlling
the total content of transporters in cells, and this could secondarily decrease
GLUT1. Since the GLUT transporter does not interact with Ubc9, and its content
in cells also does not change as Ubc9 is increased or decreased, Ubc9/sentrin is
probably having direct effects on both GLUT4 and GLUT1 transporters.

15   Other mechanisms may be at work. For example, Ubc9/sentrin may be
regulating the expression of the GLUT4 and GLUT1 genes. We do not think that
this is likely, because we know that Ubc9/sentrin interactions involve the
transporter proteins themselves, and it would be surprising if they also affected
regulatory regions of the genes. Data suggests that transporter mRNA levels do

20   not change in cells overexpressing Ubc9, and this argues against a mechanism
that involves gene regulation.

          Thus, Ubc9 itself, or sentrin attached to transporters through the catalytic
activity of Ubc9, may modify GLUT4 and GLUT1 proteins. This modification
has opposite effects leading to the stabilization and increased content of GLUT4,

25   and the breakdown and decreased content of GLUT1. The Ubc9/sentrin system
may normally have an important role in regulating the balance between the
GLUT4 and GLUT1 transporters and, thus, the degree of basal and insulin-

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stimulated glucose transport in cells. By manipulating this system purposefully, it is possible to change basal and insulin-stimulated glucose uptake. This provides for experimental systems and for treating disease states. The Ubc9/sentrin system can be used as a target for new drugs that could lower blood

5   glucose in the absence of insulin (by changing basal glucose uptake) or by augmenting insulin action (by changing insulin-stimulated glucose uptake). Similar approaches can be used in many other disease states. For example, decreasing basal glucose uptake in tissues that normally have high levels of GLUT1-mediated insulin-independent glucose transport (vascular cells and

10   nerves) could protect them from damaging effects of hyperglycemia and the development of long-term diabetes complications. In patients with strokes and decreased blood supply to regions of the brain, a drug that increased GLUT1 transporters would increase the delivery of glucose to starved cells and promote their survival, thus decreasing the extent of stroke injury and increasing the

15   function surviving neuronal cells. Many cancer cells have high levels of GLUT1 transporters. A drug that used the Ubc9/sentrin system to lower GLUT1 levels, would limit the selective advantage that these cells have for growth. The Ubc9/sentrin data presented herein not only define the system itself as a target for drug development, but also define a specific region of GLUT4 and GLUT1

20   transporters (the first 11 amino acids of the C-terminus) that has a key role in their independent regulation. This part of the transporters thus becomes a target for drug development that could involve the Ubc9/sentrin system or any other mechanism that modifies the same region of the transporters.

Gene and Cell Therapy

25   The nucleic acid constructs which encode molecules of the invention can also be used as a part of a gene or cell therapy protocol to deliver nucleic acids encoding glucose transport modulating peptides. The invention features expression vectors for transfection and expression of a glucose transport

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modulating polypeptide in particular cell types so as to reconstitute the function
of, or alternatively, modulate the function of glucose transport modulating
polypeptides in a cell. Expression constructs of glucose transport modulating
polypeptides, may be administered in any biologically effective carrier, e.g. any
5   formulation or composition capable of effectively delivering the glucose transport
modulating encoding nucleic acids to cells. Approaches include insertion of the
subject nucleic acid construct in viral vectors including recombinant retroviruses,
adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant
bacterial or eukaryotic plasmids. Viral vectors transfect cells directly; plasmid
10   DNA can be delivered with the help of, for example, cationic liposomes
(lipofectin) or derivatized (e.g. antibody conjugated) polylysine conjugates,
gramacidin S, artificial viral envelopes or other such intracellular carriers, as well
as direct injection of the gene construct or $CaPO_4$ precipitation.

A preferred approach for *in vivo* introduction of nucleic acid into a cell is
15   by use of a viral vector containing nucleic acid, e.g. a cDNA, encoding a glucose
transport modulating polypeptide. Infection of cells with a viral vector has the
advantage that a large proportion of the targeted cells can receive the nucleic acid.
Additionally, molecules encoded within the viral vector, e.g., by a cDNA
contained in the viral vector, are expressed efficiently in cells which have taken
20   up viral vector nucleic acid.

Retrovirus vectors and adeno-associated virus vectors can be used as a
recombinant gene delivery system for the transfer of exogenous genes,
particularly into humans. These vectors provide efficient delivery of genes into
cells, and the transferred nucleic acids are stably integrated into the chromosomal
25   DNA of the host. The development of specialized cell lines (termed "packaging
cells") which produce only replication-defective retroviruses has increased the
utility of retroviruses for gene therapy, and defective retroviruses are
characterized for use in gene transfer for gene therapy purposes (for a review see
Miller, A.D. (1990) *Blood* 76:271). A replication defective retrovirus can be
30   packaged into virions which can be used to infect a target cell through the use of a
helper virus by standard techniques. Protocols for producing recombinant
retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be
found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.)

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Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard
laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE
and pEM which are known to those skilled in the art. Examples of suitable
packaging virus lines for preparing both ecotropic and amphotropic retroviral
5   systems include ψCrip, ψCre, ψ2 and ψAm. Retroviruses have been used to
introduce a variety of genes and nucleic acids into many different cell types,
including epithelial cells, *in vitro* and/or *in vivo* (see for example Eglitis, et al.
(1985) *Science* 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad.
Sci. USA* 85:6460-6464; Wilson et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-
10   3018; Armentano et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber
et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry et al. (1991) *Proc.
Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury et al. (1991) *Science* 254:1802-
1805; van Beusechem et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644;
Kay et al. (1992) *Human Gene Therapy* 3:641-647; Dai et al. (1992) *Proc. Natl.*
15   *Acad. Sci. USA* 89:10892-10895; Hwu et al. (1993) *J. Immunol.* 150:4104-4115;
U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO
89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and
PCT Application WO 92/07573) which are hereby incorporated by reference.

Another viral gene delivery system useful in the present invention utilizes
20   adenovirus-derived vectors. The genome of an adenovirus can be manipulated
such that it encodes and expresses a gene product of interest but is inactivated in
terms of its ability to replicate in a normal lytic viral life cycle. See, for example,
Berkner et al. (1988) *BioTechniques* 6:616; Rosenfeld et al. (1991) *Science*
252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155. Suitable adenoviral
25   vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of
adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are known to those skilled in the art.
Recombinant adenoviruses can be advantageous in certain circumstances in that
they are not capable of infecting nondividing cells and can be used to infect a
wide variety of cell types, including epithelial cells (Rosenfeld et al. (1992) cited
30   *supra*). Furthermore, the virus particle is relatively stable and amenable to
purification and concentration, and as above, can be modified so as to affect the
spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign
DNA contained therein) is not integrated into the genome of a host cell but

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remains episomal, thereby avoiding potential problems that can occur as a result
of insertional mutagenesis in situations where introduced DNA becomes
integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying
capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases)
5   relative to other gene delivery vectors (Berkner et al. cited *supra*; Haj-Ahmand
and Graham (1986) *J. Virol.* 57:267).

Yet another viral vector system useful for delivery of the subject gene is
the adeno-associated virus (AAV). Adeno-associated virus is a naturally
occurring defective virus that requires another virus, such as an adenovirus or a
10   herpes virus, as a helper virus for efficient replication and a productive life cycle.
(For a review see Muzyczka et al. *Curr. Topics in Micro. and Immunol.* (1992)
158:97-129). It is also one of the few viruses that may integrate its DNA into
non-dividing cells, and exhibits a high frequency of stable integration (see for
example Flotte et al. (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski
15   et al. (1989) *J. Virol.* 63:3822-3828; and McLaughlin et al. (1989) *J. Virol.*
62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be
packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb.
An AAV vector such as that described in Tratschin et al. (1985) *Mol. Cell. Biol.*
5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids
20   have been introduced into different cell types using AAV vectors (see for
example Hermonat et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470;
Tratschin et al. (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford et al. (1988)
Mol. Endocrinol. 2:32-39; Tratschin et al. (1984) *J. Virol.* 51:611-619; and Flotte
et al. (1993) *J. Biol. Chem.* 268:3781-3790) which are hereby incorporated by
25   reference.

In addition to viral transfer methods, such as those illustrated above, non-
viral methods can also be employed to cause expression of a glucose transport
modulating polypeptide in the tissue of an animal. Most nonviral methods of
gene transfer rely on normal mechanisms used by mammalian cells for the uptake
30   and intracellular transport of macromolecules. In preferred embodiments,, non-
viral gene delivery systems of the present invention rely on endocytic pathways
for the uptake of the subject glucose transport modulating nucleic acid by the

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targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In a representative embodiment, a nucleic acid encoding a glucose transport modulating polypeptide can be entrapped in liposomes bearing positive

5   charges on their surface (e.g., lipofectins) and (optionally) which are tagged with antibodies against cell surface antigens of the target tissue (Mizuno et al. (1992) *No Shinkei Geka* 20:547-551; PCT publication WO91/06309; Japanese patent application 1047381; and European patent publication EP-A-43075) which are hereby incorporated by reference.

10   In clinical settings, the gene delivery systems for the therapeutic glucose transport modulating nucleic acid can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the

15   protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant nucleic acid is more limited with introduction into the animal being

20   quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by Stereotactic injection (e.g. Chen et al. (1994) *PNAS* 91: 3054-3057) which are hereby incorporated by reference.

The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a

25   slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

Antisense Therapy

30   Another aspect of the invention relates to the use of an glucose transport modulating encoding nucleic acid in "antisense" therapy. As used herein, "antisense" therapy refers to administration or *in situ* generation of oligonucleotides or their derivatives which specifically hybridize under cellular

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conditions, with cellular mRNA and/or genomic DNA so as to inhibit expression
of the encoded protein, e.g. by inhibiting transcription and/or translation. The
binding may be by conventional base pair complementarity, or, for example, in
the case of binding to DNA duplexes, through specific interactions in the major

5   groove of the double helix. In general, "antisense" therapy refers to the range of
techniques generally employed in the art, and includes any therapy which relies
on specific binding to oligonucleotide sequences.

       An antisense construct of the present invention can be delivered, for
example, as an expression plasmid which, when transcribed in the cell, produces

10   RNA which is complementary to at least a unique portion of the cellular mRNA
which encodes a glucose transport modulating peptide. Alternatively, the
antisense construct is an oligonucleotide probe which is generated *ex vivo* and
which, when introduced into the cell causes inhibition of expression by
hybridizing with the mRNA and/or genomic sequences of an glucose transport

15   modulating peptide encoding nucleic acid. Such oligonucleotide probes are
preferably modified oligonucleotide which are resistant to endogenous nucleases,
e.g. exonucleases and/or endonucleases, and is therefore stable *in vivo*.
Exemplary nucleic acid molecules for use as antisense oligonucleotides are
phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see

20   also U.S. Patents 5,176,996; 5,264,564; and 5,256,775). Additionally, general
approaches to constructing oligomers useful in antisense therapy have been
reviewed, for example, by Van der Krol et al. (1988) *Biotechniques* 6:958-976;
and Stein et al. (1988) *Cancer Res* 48:2659-2668 which are hereby incorporated
by reference.

25   The antisense constructs of the present invention, by antagonizing the
normal biological activity of the glucose transport modulating peptide, can be
used in the manipulation of glucose homeostasis, both *in vitro* and *in vivo*.
**Production of Fragments and Analogs of Ubc9, GLUT4 and GLUT1 and
other useful sequences**

30   The inventor has discovered that the interaction of GLUT4 and GLUT1
with Ubc9 regulates glucose uptake in the cell. One skilled in the art can alter the
structure of GLUT4, GLUT1, and Ubc9, e.g., by producing fragments or analogs,
and test the newly produced structures for activity. The methods can also be used

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to make GLUT4, GLUT1, or Ubc9 binding peptides from other, e.g. randomly
generated, sequences. Examples of prior art methods which allow the production
and testing of fragments and analogs are discussed below. These, or other
methods, can be used to make and screen fragments and analogs of a glucose
5   transport modulator.

Generation of Fragments

Fragments of a protein can be produced in several ways, e.g.,
recombinantly, by proteolytic digestion, or by chemical synthesis. Internal or
terminal fragments of a polypeptide can be generated by removing one or more
10   nucleotides from one end (for a terminal fragment) or both ends (for an internal
fragment) of a nucleic acid which encodes the polypeptide. Expression of the
mutagenized DNA produces polypeptide fragments. Digestion with "end-
nibbling" endonucleases can thus generate DNA's which encode an array of
fragments. DNA's which encode fragments of a protein can also be generated by
15   random shearing, restriction digestion or a combination of the above-discussed
methods.

Fragments can also be chemically synthesized using techniques known in
the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For
example, peptides of the present invention may be arbitrarily divided into
20   fragments of desired length with no overlap of the fragments, or divided into
overlapping fragments of a desired length.

**Generation of Analogs: Production of Altered DNA and Peptide Sequences
by Random Methods**

Amino acid sequence variants of a protein can be prepared by random
25   mutagenesis of DNA which encodes a protein or a particular domain or region of
a protein. Useful methods include PCR mutagenesis and saturation mutagenesis.
A library of random amino acid sequence variants can also be generated by the
synthesis of a set of degenerate oligonucleotide sequences. (Methods for
screening proteins in a library of variants are described elsewhere herein.)
30   **PCR Mutagenesis**

In PCR mutagenesis, reduced Taq polymerase fidelity is used to introduce
random mutations into a cloned fragment of DNA (Leung et al., 1989, *Technique*
1:11-15). This is a very powerful and relatively rapid method of introducing

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random mutations. The DNA region to be mutagenized is amplified using the polymerase chain reaction (PCR) under conditions that reduce the fidelity of DNA synthesis by Taq DNA polymerase, e.g., by using a dGTP/dATP ratio of five and adding Mn^{2+} to the PCR reaction. The pool of amplified DNA

5   fragments are inserted into appropriate cloning vectors to provide random mutant libraries.

Saturation Mutagenesis

Saturation mutagenesis allows for the rapid introduction of a large number of single base substitutions into cloned DNA fragments (Mayers et al., 1985,

10   *Science* 229:242). This technique includes generation of mutations, e.g., by chemical treatment or irradiation of single-stranded DNA *in vitro*, and synthesis of a complimentary DNA strand. The mutation frequency can be modulated by modulating the severity of the treatment, and essentially all possible base substitutions can be obtained. Because this procedure does not involve a genetic

15   selection for mutant fragments both neutral substitutions, as well as those that alter function, are obtained. The distribution of point mutations is not biased toward conserved sequence elements.

Degenerate Oligonucleotides

A library of homologs can also be generated from a set of degenerate

20   oligonucleotide sequences. Chemical synthesis of a degenerate sequences can be carried out in an automatic DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The synthesis of degenerate oligonucleotides is known in the art (see for example, Narang, SA (1983) *Tetrahedron* 39:3; Itakura et al. (1981) *Recombinant DNA, Proc 3rd Cleveland*

25   *Sympos. Macromolecules*, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al. (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al. (1984) *Science* 198:1056; Ike et al. (1983) *Nucleic Acid Res.* 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) *Science* 249:386-390; Roberts et al. (1992) *PNAS* 89:2429-2433; Devlin et

30   al. (1990) *Science* 249: 404-406; Cwirla et al. (1990) *PNAS* 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815) which are hereby incorporated by reference.

27.

Generation of Analogs: Production of Altered DNA and Peptide Sequences by Directed Mutagenesis

Non-random or directed, mutagenesis techniques can be used to provide specific sequences or mutations in specific regions. These techniques can be used to create variants which include, e.g., deletions, insertions, or substitutions, of residues of the known amino acid sequence of a protein. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conserved amino acids and then with more radical choices depending upon results achieved, (2) deleting the target residue, or (3) inserting residues of the same or a different class adjacent to the located site, or combinations of options 1-3.

Alanine Scanning Mutagenesis

Alanine scanning mutagenesis is a useful method for identification of certain residues or regions of the desired protein that are preferred locations or domains for mutagenesis, Cunningham and Wells (*Science* 244:1081-1085, 1989). In alanine scanning, a residue or group of target residues are identified (e.g., charged residues such as Arg, Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine). Replacement of an amino acid can affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions are then refined by introducing further or other variants at or for the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to optimize the performance of a mutation at a given site, alanine scanning or random mutagenesis may be conducted at the target codon or region and the expressed desired protein subunit variants are screened for the optimal combination of desired activity.

Oligonucleotide-Mediated Mutagenesis

Oligonucleotide-mediated mutagenesis is a useful method for preparing substitution, deletion, and insertion variants of DNA, see, e.g., Adelman et al., (*DNA* 2:183, 1983). Briefly, the desired DNA is altered by hybridizing an oligonucleotide encoding a mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered

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or native DNA sequence of the desired protein. After hybridization, a DNA
polymerase is used to synthesize an entire second complementary strand of the
template that will thus incorporate the oligonucleotide primer, and will code for
the selected alteration in the desired protein DNA. Generally, oligonucleotides of

5   at least 25 nucleotides in length are used. An optimal oligonucleotide will have
12 to 15 nucleotides that are completely complementary to the template on either
side of the nucleotide(s) coding for the mutation. This ensures that the
oligonucleotide will hybridize properly to the single-stranded DNA template
molecule. The oligonucleotides are readily synthesized using techniques known

10   in the art such as that described by Crea et al. (*Proc. Natl. Acad. Sci.* USA, 75:
5765[1978]) which is hereby incorporated by reference.

        Cassette Mutagenesis

        Another method for preparing variants, cassette mutagenesis, is based on
the technique described by Wells et al. (*Gene*, 34:315[1985]). The starting

15   material is a plasmid (or other vector) which includes the protein subunit DNA to
be mutated. The codon(s) in the protein subunit DNA to be mutated are
identified. There must be a unique restriction endonuclease site on each side of
the identified mutation site(s). If no such restriction sites exist, they may be
generated using the above-described oligonucleotide-mediated mutagenesis

20   method to introduce them at appropriate locations in the desired protein subunit
DNA. After the restriction sites have been introduced into the plasmid, the
plasmid is cut at these sites to linearize it. A double-stranded oligonucleotide
encoding the sequence of the DNA between the restriction sites but containing
the desired mutation(s) is synthesized using standard procedures. The two

25   strands are synthesized separately and then hybridized together using standard
techniques. This double-stranded oligonucleotide is referred to as the cassette.
This cassette is designed to have 3' and 5' ends that are comparable with the ends
of the linearized plasmid, such that it can be directly ligated to the plasmid. This
plasmid now contains the mutated desired protein subunit DNA sequence.

30   **Combinatorial Mutagenesis**

        Combinatorial mutagenesis can also be used to generate mutants. E.g.,
the amino acid sequences for a group of homologs or other related proteins are
aligned, preferably to promote the highest homology possible. All of the amino

29.

acids which appear at a given position of the aligned sequences can be selected to
create a degenerate set of combinatorial sequences. The variegated library of
variants is generated by combinatorial mutagenesis at the nucleic acid level, and
is encoded by a variegated gene library. For example, a mixture of synthetic
5   oligonucleotides can be enzymatically ligated into gene sequences such that the
degenerate set of potential sequences are expressible as individual peptides, or
alternatively, as a set of larger fusion proteins containing the set of degenerate
sequences.

Analogs

10   Analogs can differ from naturally occurring glucose transport modulators
in amino acid sequence or in ways that do not involve sequence, or both. Non-
sequence modifications include in vivo or in vitro chemical derivatization of
glucose transport modulators. Non-sequence modifications include changes in
acetylation, methylation, phosphorylation, carboxylation, or glycosylation.

15   Preferred analogs of Ubc9, GLUT4, and GLUT1 (or fragments thereof)
have sequences which differ from the wild-type sequence by one or more
conservative amino acid substitutions or by one or more non-conservative amino
acid substitutions, deletions, or insertions which do not abolish the glucose
transport modulator biological activity. Conservative substitutions typically
20   include the substitution of one amino acid for another with similar characteristics,
e.g., substitutions within the following groups: valine, glycine; glycine, alanine;
valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine;
serine, threonine; lysine, arginine; and phenylalanine, tyrosine. Other
conservative substitutions can be taken from the table below.

30.

TABLE 1
CONSERVATIVE AMINO ACID REPLACEMENTS

For Amino Acid	Code	Replace with any of
Alanine	A	D-Ala, Gly, beta-Ala, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile,Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, β-Ala, Acp
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, Leu, D-Leu, Met, D-Met
Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val
Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, cis-3,4, or 5-phenylproline
Proline	P	D-Pro, L-I-thioazolidine-4-carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met

5

Other analogs within the invention are those with modifications which increase peptide stability; such analogs may contain, for example, one or more non-peptide bonds (which replace the peptide bonds) in the peptide sequence.

31.

Also included are: analogs that include residues other than naturally occurring L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g., β or γ amino acids; and cyclic analogs.

Peptide Mimetics

5   The invention also provides mimetics, e.g. peptide or non-peptide mimetics, of GLUT4, GLUT4 C-terminal peptide fragment, GLUT4 moieties, GLUT1, GLUT1 C-terminal peptide fragment, GLUT1 moieties, Ubc9, or Ubc9 moieties. Peptide mimetics can modulate the interaction of GLUT4 or GLUT1 to Ubc9. The critical residues of a subject glucose transport modulator polypeptide

10   which are involved in molecular recognition of a polypeptide can be determined and used to generate GLUT4-derived or GLUT1-derived peptidomimetics which competitively or noncompetitively inhibit the interaction between GLUT4 or GLUT1 with Ubc9 (see, for example, "Peptide inhibitors of human papillomavirus protein binding to retinoblastoma gene protein" European patent

15   applications EP-412,762A and EP-B31,080A). For example, scanning mutagenesis can be used to map the amino acid residues of a particular GLUT4 polypeptide involved in interacting with Ubc9, and peptidomimetic compounds (e.g. diazepine or isoquinoline derivatives) generated which mimic those residues involved in interacting with Ubc9. These products can inhibit binding of a

20   GLUT4 or GLUT1 polypeptide to a ligand and thereby interfere with the function of GLUT4 or GLUT1 or Ubc9.

Non-hydrolyzable peptide analogs of critical residues can be generated using benzodiazepine (e.g., see Freidinger et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988),

25   azepine (e.g., see Huffman et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gama lactam rings (Garvey et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al. (1986) *J Med Chem* 29:295; and Ewenson et al.

30   in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), β-turn dipeptide cores (Nagai et al. (1985) *Tetrahedron Lett* 26:647; and Sato et al. (1986) *J Chem Soc Perkin Trans* 1:1231), and β-aminoalcohols (Gordon et al. (1985) *Biochem*

32.

*Biophys Res Commun*126:419; and Dann et al. (1986) *Biochem Biophys Res Commun* 134:71) hereby incorporated by reference.

Accordingly, the polypeptides, nucleic acids, and other compounds of the invention are useful in therapeutic, diagnostic, and research contexts. The polypeptides and nucleic acids of the invention can be formulated for a variety of loads of administration, including systemic and topical or localized administration. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous for injection, the polypeptides and nucleic acids of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the polypeptides and nucleic acids may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included in the invention.

The polypeptides and nucleic acids can be administered orally, or by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives, and detergents. Transmucosal administration may be through nasal sprays or using suppositories. For oral administration, the polypeptides and nucleic acids are formulated into conventional oral administration forms such as capsules, tablets, and tonics. For topical administration, the oligomers of the invention are formulated into ointments, salves, gels, or creams as known in the art.

Methods of Identifying Modulators of Glucose Uptake

The invention provides methods for evaluating a compound for the ability to modulate glucose transport. One method includes: providing the GLUT4 C-Terminal tail peptide or a GLUT4 binding moiety of Ubc9, contacting the compound with the GLUT4 tail peptide or the Ubbc9 moiety, determining if the compound binds the GLUT4 tail peptide or the Ubc9 moiety, the binding of the compound being indicative of its ability to inhibit the interaction.

Primary High-Through-Put Methods for Screening Libraries of Peptide Fragments or Homologs

33.

Libraries of compounds can also be screened to determine whether any members of the library have the desired glucose transport modulating activity, and, if so, to identify the most active species. Various techniques are known in the art for screening generated mutant gene products. Techniques for screening

5   large gene libraries often include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the genes under conditions in which detection of a desired activity, e.g., in this case, the interaction, e.g., binding, of GLUT4 to a vesicle coating peptide, or the interaction, e.g., binding of a candidate polypeptide with a

10   GLUT4 binding fragment or a vesicle coating binding fragment facilitate relatively easy isolation of the vector encoding the gene whose product was detected. Each of the techniques described below is amenable to high through-put analysis for screening large numbers of sequences created, e.g., by random mutagenesis techniques.

15   **Two Hybrid Systems**

Two hybrid assays such as the system described above (as with the other screening methods described herein), can be used to identify fragments or analogs which can bind to the GLUT4, GLUT1 or Ubc9 moieties. These may include agonists, superagonists, and antagonists. For instance, a GLUT4 moiety can be

20   used as the bait protein and the library of variants of Ubc9 are used as fish fusion proteins. Thereby, identifying fragments or analogs of Ubc9 which bind to the GLUT4 moiety.

Display Libraries

In one approach to screening assays, the candidate peptides are displayed

25   on the surface of a cell or viral particle, and the ability of particular cells or viral particles to bind an appropriate receptor protein via the displayed product is detected in a "panning assay". For example, the gene library can be cloned into the gene for a surface membrane protein of a bacterial cell, and the resulting fusion protein detected by panning (Ladner et al., WO 88/06630; Fuchs et al.

30   (1991) *Bio/Technology* 9:1370-1371; and Goward et al. (1992) *TIBS* 18:136-140) which are hereby incorporated by reference. In a similar fashion, a detectably labeled ligand can be used to score for potentially functional peptide homologs. Fluorescently labeled ligands, e.g., receptors, can be used to detect homolog

34.

which retain ligand-binding activity. The use of fluorescently labeled ligands, allows cells to be visually inspected and separated under a fluorescence microscope, or, where the morphology of the cell permits, to be separated by a fluorescence-activated cell sorter.

5   A gene library can be expressed as a fusion protein on the surface of a viral particle. For instance, in the filamentous phage system, foreign peptide sequences can be expressed on the surface of infectious phage, thereby conferring two significant benefits. First, since these phage can be applied to affinity matrices at concentrations well over 10^{13} phage per milliliter, a large number of
10   phage can be screened at one time. Second, since each infectious phage displays a gene product on its surface, if a particular phage is recovered from an affinity matrix in low yield, the phage can be amplified by another round of infection. The group of almost identical *E. coli* filamentous phages M13, fd., and f1 are most often used in phage display libraries. Either of the phage gIII or gVIII coat
15   proteins can be used to generate fusion proteins without disrupting the ultimate packaging of the viral particle. Foreign epitopes can be expressed at the NH_2-terminal end of pIII and phage bearing such epitopes recovered from a large excess of phage lacking this epitope (Ladner et al. PCT publication WO 90/02909; Garrard et al., PCT publication WO 92/09690; Marks et al. (1992) *J.*
20   *Biol. Chem.* 267:16007-16010; Griffiths et al. (1993) *EMBO J* 12:725-734; Clackson et al. (1991) *Nature* 352:624-628; and Barbas et al. (1992) *PNAS* 89:4457-4461) which are hereby incorporated by reference.

A common approach uses the maltose receptor of *E. coli* (the outer membrane protein, LamB) as a peptide fusion partner (Charbit et al. (1986)
25   *EMBO* 5, 3029-3037). Oligonucleotides have been inserted into plasmids encoding the LamB gene to produce peptides fused into one of the extracellular loops of the protein. These peptides are available for binding to ligands, e.g., to antibodies, and can elicit an immune response when the cells are administered to animals. Other cell surface proteins, e.g., OmpA (Schorr et al. (1991) *Vaccines*
30   *91*, pp. 387-392), PhoE (Agterberg, et al. (1990) *Gene* 88, 37-45), and PAL (Fuchs et al. (1991) *Bio/Tech* 9, 1369-1372), as well as large bacterial surface structures have served as vehicles for peptide display. Peptides can be fused to pilin, a protein which polymerizes to form the pilus-a conduit for interbacterial

35.

exchange of genetic information (Thiry et al. (1989) *Appl. Environ. Microbiol.* 55, 984-993). Because of its role in interacting with other cells, the pilus provides a useful support for the presentation of peptides to the extracellular environment. Another large surface structure used for peptide display is the

5   bacterial motive organ, the flagellum. Fusion of peptides to the subunit protein flagellin offers a dense array of may peptides copies on the host cells (Kuwajima et al. (1988) *Bio/Tech.* 6, 1080-1083). Surface proteins of other bacterial species have also served as peptide fusion partners. Examples include the *Staphylococcus* protein A and the outer membrane protease IgA of *Neisseria*

10   (Hansson et al. (1992) *J. Bacteriol.* 174, 4239-4245 and Klauser et al. (1990) *EMBO J.* 9, 1991-1999) which are hereby incorporated by reference.

In the filamentous phage systems and the LamB system described above, the physical link between the peptide and its encoding DNA occurs by the containment of the DNA within a particle (cell or phage) that carries the peptide

15   on its surface. Capturing the peptide captures the particle and the DNA within. An alternative scheme uses the DNA-binding protein LacI to form a link between peptide and DNA (Cull *et al.* (1992) *PNAS USA* 89:1865-1869). This system uses a plasmid containing the LacI gene with an oligonucleotide cloning site at its 3'-end. Under the controlled induction by arabinose, a LacI-peptide fusion

20   protein is produced. This fusion retains the natural ability of LacI to bind to a short DNA sequence known as LacO operator (LacO). By installing two copies of LacO on the expression plasmid, the LacI-peptide fusion binds tightly to the plasmid that encoded it. Because the plasmids in each cell contain only a single oligonucleotide sequence and each cell expresses only a single peptide sequence,

25   the peptides become specifically and stably associated with the DNA sequence that directed its synthesis. The cells of the library are gently lysed and the peptide-DNA complexes are exposed to a matrix of immobilized receptor to recover the complexes containing active peptides. The associated plasmid DNA is then reintroduced into cells for amplification and DNA sequencing to

30   determine the identity of the peptide ligands. As a demonstration of the practical utility of the method, a large random library of dodecapeptides was made and selected on a monoclonal antibody raised against the opioid peptide dynorphin B. A cohort of peptides was recovered, all related by a consensus sequence

36.

corresponding to a six-residue portion of dynorphin B. (Cull et al. (1992) *Proc.
Natl. Acad. Sci. U.S.A.* 89-1869) which is hereby incorporated by reference

This scheme, sometimes referred to as peptides-on-plasmids, differs in
two important ways from the phage display methods. First, the peptides are

5   attached to the C-terminus of the fusion protein, resulting in the display of the
library members as peptides having free carboxy termini. Both of the filamentous
phage coat proteins, pIII and pVIII, are anchored to the phage through their C-
termini, and the guest peptides are placed into the outward-extending N-terminal
domains. In some designs, the phage-displayed peptides are presented right at the

10   amino terminus of the fusion protein. (Cwirla, et al. (1990) *Proc. Natl. Acad. Sci.
U.S.A.* 87, 6378-6382) A second difference is the set of biological biases
affecting the population of peptides actually present in the libraries. The LacI
fusion molecules are confined to the cytoplasm of the host cells. The phage coat
fusions are exposed briefly to the cytoplasm during translation but are rapidly

15   secreted through the inner membrane into the periplasmic compartment,
remaining anchored in the membrane by their C-terminal hydrophobic domains,
with the N-termini, containing the peptides, protruding into the periplasm while
awaiting assembly into phage particles. The peptides in the LacI and phage
libraries may differ significantly as a result of their exposure to different

20   proteolytic activities. The phage coat proteins require transport across the inner
membrane and signal peptidase processing as a prelude to incorporation into
phage. Certain peptides exert a deleterious effect on these processes and are
underrepresented in the libraries (Gallop et al. (1994) *J. Med. Chem.* 37(9):1233-
1251). These particular biases are not a factor in the LacI display system.

25   The number of small peptides available in recombinant random libraries is
enormous. Libraries of 10^7-10^9 independent clones are routinely prepared.
Libraries as large as 10^{11} recombinants have been created, but this size
approaches the practical limit for clone libraries. This limitation in library size
occurs at the step of transforming the DNA containing randomized segments into

30   the host bacterial cells. To circumvent this limitation, an *in vitro* system based on
the display of nascent peptides in polysome complexes has recently been
developed. This display library method has the potential of producing libraries 3-
6 orders of magnitude larger than the currently available phage/phagemid or

37.

plasmid libraries. Furthermore, the construction of the libraries, expression of the peptides, and screening, is done in an entirely cell-free format.

In one application of this method (Gallop et al. (1994) *J. Med. Chem.* 37(9):1233-1251), a molecular DNA library encoding 10^{12} decapeptides was constructed and the library expressed in an *E. coli* S30 *in vitro* coupled transcription/translation system. Conditions were chosen to stall the ribosomes on the mRNA, causing the accumulation of a substantial proportion of the RNA in polysomes and yielding complexes containing nascent peptides still linked to their encoding RNA. The polysomes are sufficiently robust to be affinity purified on immobilized receptors in much the same way as the more conventional recombinant peptide display libraries are screened. RNA from the bound complexes is recovered, converted to cDNA, and amplified by PCR to produce a template for the next round of synthesis and screening. The polysome display method can be coupled to the phage display system. Following several rounds of screening, cDNA from the enriched pool of polysomes was cloned into a phagemid vector. This vector serves as both a peptide expression vector, displaying peptides fused to the coat proteins, and as a DNA sequencing vector for peptide identification. By expressing the polysome-derived peptides on phage, one can either continue the affinity selection procedure in this format or assay the peptides on individual clones for binding activity in a phage ELISA, or for binding specificity in a completion phage ELISA (Barret, et al. (1992) *Anal. Biochem* 204,357-364). To identify the sequences of the active peptides one sequences the DNA produced by the phagemid host.

Secondary Screens

The high through-put assays described above can be followed by secondary screens in order to identify further biological activities which will, e.g., allow one skilled in the art to differentiate agonists from antagonists. The type of a secondary screen used will depend on the desired activity that needs to be tested. For example, an assay can be developed in which the ability to inhibit an interaction between a protein of interest, e.g. GLUT4, and its respective ligand, e.g. Ubc9, can be used to identify antagonists from a group of peptide fragments isolated though one of the primary screens described above.

38.

Therefore, methods for generating fragments and analogs and testing them for activity are known in the art. Once the core sequence of interest is identified, it is routine to perform for one skilled in the art to obtain analogs and fragments.

Drug Screening Assays

5   By making available purified and recombinant-glucose transport modulator polypeptides, the present invention provides assays which can be used to screen for drugs which are either agonists or antagonists of the normal cellular function, in this case, of GLUT4, GLUT1 and Ub9. In one embodiment, the assay evaluates the ability of a compound to modulate binding between a GLUT4

10   polypeptide and a naturally occurring ligand, e.g., Ubc9. A variety of assay formats will suffice and, in light of the present inventions, will be comprehended by skilled artisan.

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the

15   number of compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover,

20   the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with other proteins or change in enzymatic properties of the molecular target.

25   **Examples**

Example 1: Interaction of Ubc9 with the C-Terminal Region of the GLUT4 glucose transporter in the yeast two-hybrid system.

Table 2:

GLUT4 Fragment	Extent of Ubc9 Binding
N-Terminus	-
Cytoplasmic Loop	+/-

39.

C-Terminus	+++

Full-length Ubc9 was isolated from a mouse fat cell cDNA expression library as a cDNA encoding a protein that binds with high specificity to the C-terminal intracellular portion of the GLUT4 glucose transporter using the yeast
5   two-hybrid cloning system. Ubc9 was the most abundant GLUT4-interactive clone identified in this screening (5 of 10 total clones isolated). The capacity of Ubc9 to bind to the three major intracellular domains of the GLUT4 transporter was then tested in a further analysis using the yeast two-hybrid system, as shown in Table 2. There was strong (+++) binding of Ubc9 to the C-terminus of
10   GLUT4, very weak (+/-) binding to the cytoplasmic loop in the mid-portion of GLUT4, and no detectable binding (-) to the N-terminal intracellular portion of GLUT4. This demonstrates specificity of Ubc9 for binding to the C-terminal region of GLUT4, as compared to other intracellular domains of the transporter.
        Example 2.   Binding of recombinant Ubc9 to recombinant GLUT4 C-terminal
15   peptide and its inhibition by a synthetic peptide corresponding to amino acids 467-477 of GLUT4.

        A recombinant GLUT4 peptide corresponding to the C-terminal intracellular portion of the transporter (amino acids 467-509) was generated in *E. coli* and immobilized by linking to a resin. Full-length Ubc9 protein fused to
20   glutathione S-transferase (GST) (generated in *E. coli*) was interacted with the immobilized GLUT4; the resin was washed to remove non-specifically bound protein, the GLUT4 peptide and any proteins attached to it were eluted from the resin with an imidazole buffer, the eluted proteins were resolved by gel electrophoresis; and the amount of Ubc9 that was bound to GLUT4 was
25   determined by immunoblotting with GST antibody.

        A control was included, in which the GLUT4 peptide was not present. Specific binding of Ubc9 to the GLUT4 C-terminal region was observed. This confirms the binding interaction shown with the two-hybrid system in Table 2, and it demonstrates that the interaction is direct, without a requirement for other
30   intervening proteins. Inhibition of Ubc9 binding to GLUT4 was observed when a synthetic peptide corresponding to amino acids 467-477 of the transporter was

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added (concentrations of 0.1mM, 1mM, and 5mM of the synthetic peptide). This shows that Ubc9 binds to a segment including the first 11 amino acids of the intracellular C-terminal portion of GLUT4 (i.e., amino acids 467-477).

Example 3.   Binding of recombinant Ubc9 to different recombinant glucose
5   transporter protein constructs

Recombinant glucose transporter peptides containing several different C-terminal sequences were generated in *E. coli* and immobilized by linking to a resin. The specific constructs included a recombinant protein lacking transporter sequence as a control, the full intracellular C-terminal portion of GLUT4, the
10   intracellular C-terminal portion of the GLUT1 glucose transporter, a truncated intracellular C-terminal portion of GLUT4 lacking the first 11 amino acids (amino acids 467-477), and a mutated GLUT4 C-terminal portion of GLUT4 in which leucine residues at amino acids 489 and 490 were changed to alanine and serine, respectively. Full-length Ubc9 protein fused to glutathione S-transferase
15   (GST) (generated in *E. coli*) was interacted with the immobilized glucose transporter constructs; the resin was washed to remove non-specifically bound protein; the glucose transporter peptides and any proteins attached to them were eluted from the resin with an imidazole buffer; the eluted proteins were resolved by gel electrophoresis; and the amount of Ubc9 that was bound to the transporter
20   constructs was determined by immunoblotting with GST antibody.

A control was included in which the glucose transporter peptide was not present. Ubc9 was observed to bind to the C-terminal regions of GLUT4, GLUT1, and the mutant GLUT4 (LL→AS). This demonstrates that the LL sequence at amino acids 489-490 is not involved in Ubc9 binding to the
25   transporter. Decreased binding of Ubc9 to a GLUT 4 C-terminal peptide lacking the first 11 amino acids was observed, confirming the requirement of the first 11 amino acids of the C-terminal portion of the transporter for effective Ubc9 binding. The binding of Ubc9 to the various transporter constructs is inhibited by the addition of a synthetic peptide corresponding to amino acids 467-477.

30   **Example 4.**   Binding of recombinant Ubc9 to native GLUT4 glucose transporter in membrane fractions from cultured L6 myoblasts.

Recombinant full-length Ubc9 protein fused to glutathione S-transferase (GST) was generated in *E. coli* and immobilized by linking to a resin.

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Solubilized extracts of L6 myoblasts (cultured skeletal muscle precursor cells) were prepared that were enriched in either plasma membrane (PM) components or low density microsomal (LDM) components. These cellular fractions were interacted with the immobilized Ubc9; the resin was washed to remove non-
5   specifically bound proteins; the Ubc9 and any proteins attached to it were eluted from the resin with a glutathione buffer; the eluted proteins were resolved by gel electrophoresis; and the amount of native GLUT4 glucose transporter in the cell fractions that was bound to Ubc9 was determined by immunoblotting with GLUT4 antibody.

10   Negative controls without Ubc9 demonstrated the requirement of Ubc9 for GLUT4 binding. Both LDM and PM fractions interacted with immobilized Ubc9. The native GLUT4 glucose transporter in both of these cellular fractions binds specifically to the Ubc9 protein, although PM GLUT4 has a stronger binding affinity for the Ubc9 protein than does LDM GLUT4.

15   **Example 5.** Binding of recombinant Ubc9 to native GLUT3 glucose transporter in membrane fractions from cultured L6 myoblasts.

Recombinant full-length Ubc9 protein fused to glutathione S-transferase (GST) was generated in *E. coli* and immobilized by linking to a resin. Solubilized extracts of L6 myoblasts (cultured skeletal muscle precursor cells)
20   were prepared that were enriched in either plasma membrane (PM) components or low density microsomal (LDM) components. These cellular fractions were interacted with the immobilized Ubc9; the resin was washed to remove non-specifically bound proteins; the Ubc9 and any proteins attached to it were eluted from the resin with a glutathione buffer; the eluted proteins were resolved by gel
25   electrophoresis; and the amount of native GLUT3 glucose transporter in the cell fractions that was bound to Ubc9 was determined by immunoblotting with GLUT3 antibody.

Negative controls without Ubc9 showed no binding to GLUT3. LDM and PM fractions of GLUT3 demonstrated no binding to Ubc9, despite the abundance
30   of GLUT3 glucose transporter in the LDM fraction (confirmed by independent measures). Thus, the binding of GLUT4 and GLUT1 to Ubc9 demonstrated previous examples is specific, and all glucose transporter isoforms do not bind to Ubc9. A positive GLUT3 control was also included.

42.

Example 6.   Effects of increased cellular content of Ubc9 on glucose uptake in
L6 myoblasts.

       L6 myoblasts were transfected with the pCRT3 expression vector
containing cDNAs corresponding to full-length Ubc9 or to a mutated Ubc9 in

5   which amino acid 93 was changed from cysteine to alanine. This mutation is in
the presumed active site region of Ubc9 and is expected to result in a catalytically
inactive Ubc9 enzyme. As a control, L6 myoblasts were similarly transfected
with the pCR3 expression vector lacking a Ubc9 cDNA. Myoblasts expressing
increased levels of the different Ubc9 constructs were isolated by growth in

10   selective medium, and several cell clones were isolated that had an approximately
4-fold increased in Ubc9 protein, as compared to cells transfected with the control
vector. The level of Ubc9 expression in these cells was determined both by
quantitative polymerase chain reaction analysis of mRNA and by immunoblotting
with Ubc9 antibody. Basal and insulin-stimulated uptake of glucose in the

15   various cell clones was then determined using a standard 2-deoxyglucose uptake
assay.

       As shown by the open bars in Figure 1, insulin resulted in an
approximately 50% increase in glucose uptake in the cells transfected with the
control vector. When the cellular content of Ubc9 was increased (cross-hatched

20   bars), basal glucose uptake decreased by approximately 70%, whereas the fold-
stimulation of glucose uptake by insulin increased to approximately 300% of
basal. In the cells transfected with the alanine93 mutant Ubc9, basal glucose
uptake was markedly increased (approximately 80% above the controls), whereas
the fold-increase in glucose uptake in response to insulin was decreased by 27%.

25   These data indicate inhibition of basal and augmentation of insulin-stimulated
glucose uptake as a consequence of increased cellular content of normal Ubc9.
Increased cellular content of the catalytically inactive mutant form of Ubc9 has
the opposite effect, leading to increased basal and decreased insulin stimulation
of glucose uptake. This can be explained by competition of the mutant Ubc9

30   protein with the native protein for binding to glucose transporters.

Example 7.   Effects of increased cellular content of Ubc9 on GLUT4 glucose
transporter content in L6 myoblasts.

43.

L6 myoblasts were transfected with the pCR3 expression vector containing cDNAs corresponding to full-length Ubc9 or to a mutated Ubc9 in which amino acid 93 was changed from cysteine to alanine. This mutation is in the presumed active site region of Ubc9 and is expected to result in a catalytically

5   inactive Ubc9 enzyme. As a control, L6 myoblasts were similarly transfected with the pCR3 expression vector lacking a Ubc9 cDNA. Myoblasts expressing increased levels of the different Ubc9 constructs were isolated by growth in selective medium, and several cell clones were isolated that had an approximately 4-fold increased in Ubc9 protein, as compared to cells transfected with the control

10   vector. The level of Ubc9 expression in these cells was determined both by quantitative polymerase chain reaction analysis of mRNA and by immunoblotting with Ubc9 antibody. The cellular content of the GLUT4 glucose transporter in the various cell clones was then determined by preparing cellular extracts, resolving the cellular proteins by gel electrophoresis, and immunoblotting with

15   GLUT4 antibody.

It was observed that in normal L6 myoblasts, there is a low level of GLUT4 glucose transporter protein present. A similar amount of GLUT4 was observed in two cell clones transfected with the PCR3 vector lacking a Ubc9 cDNA. In three cell clones expressing similar levels of the alanine 93

20   catalytically inactive Ubc9 protein, it was observed that GLUT4 levels were equivalent to that of the controls. Thus, the increase in GLUT4 induced by increased Ubc9 expression requires a catalytically active form of the Ubc9 enzyme.

Example 8. Effects of increased cellular content of Ubc9 on GLUT1 glucose

25   transporter Content in L6 myoblasts.

L6 myoblasts were transfected with the pCR3 expression vector containing cDNAs corresponding to full-length Ubc9 or to a mutated Ubc9 in which amino acid 93 was changed from cysteine to alanine. This mutation is in the presumed active site region of Ubc9 and is expected to result in a catalytically

30   inactive Ubc9 enzyme. As a control, L6 myoblasts were similarly transfected with the pCR3 expression vector lacking a Ubc9 cDNA. Myoblasts expressing increased levels of the different Ubc9 constructs were isolated by growth in selective medium, and several cell clones were isolated that had an approximately

44.

4-fold increased in Ubc9 protein, as compared to cells transfected with the control
vector. The level of Ubc9 expression in these cells was determined both by
quantitative polymerase chain reaction analysis of mRNA and by immunoblotting
with Ubc9 antibody. The cellular content of the GLUT1 glucose transporter in

5   the various cell clones was then determined by preparing cellular extracts,
resolving the cellular proteins by gel electrophoresis, and immunoblotting with
GLUT1 antibody.

The content of GLUT1 glucose transporter protein present in two L6
myoblast cones transfected with the pCR3 vector lacking a Ubc9 cDNA were

10   used as a control. Three cell clones, with an approximately four-fold increase of
Ubc9 content over the controls, were observed to have an approximately 60%
decrease in the cellular content of the GLUT1 glucose transporter. In another set
of three cell clones, expressing similar levels of the alanine93 catalytically
inactive Ubc9 protein, GLUT1 levels were observed to be increased by 30% in

15   these cells in compared with the controls. Thus, Ubc9 has opposite effects on
GLUT1 and GLUT4 content. Normal Ubc9 leads to decreased GLUT1 cellular
content and increased GLUT4 content. Expression of a catalytically inactive
mutant Ubc9 results in increased GLUT1 cellular content above the level of
normal cells. This can be explained by competition of the mutant form of the

20   enzyme with the native Ubc9 enzyme for binding to GLUT1, and it demonstrates
the requirement for Ubc9 catalytic activity for its suppressive effect on the
cellular content of GLUT1.

Example 9. Attachment of Sentrin to glucose transporters in normal cells.

There is evidence that the catalytic activity of Ubc9 results in the covalent

25   attachment of a peptide designated sentrin to other proteins. To investigate
whether sentrin is attached to glucose transporters in normal cells, solubilized
extracts of various fractions of differentiated 3T3-L1 adipocytes were prepared
and subjected to immunoprecipitation with GLUT4 or GLUT1 glucose
transporter antibodies. The resulting immunoprecipitates were resolved by gel

30   electrophoresis, and the presence of sentrin attached to the glucose transporters
was determined by immunoblotting with sentrin antibody.

Sentrinized glucose transporters (i.e., glucose transporters with sentrin
attached) were evident as an approximately 85 kD band in low density

45.

microsome fractions 3T3-L1 adipocytes, high density microsome fractions, and plasma membrane fractions. Negative controls were done , in which antibodies against proteins other than GLUT4 and GLUT 1 were used for the immunoprecipitation step, or non-precipitating conditions were used. These data

5   demonstrate the specific, covalent linkage of sentrin to the GLUT4 and GLUT 1 glucose transporters, and they support the conclusion that Ubc9 binding to glucose transporters is followed by sentrin attachment to the transporters, mediated by the catalytic activity of Ubc9. Thus, the observed effects of Ubc9 on glucose transporter function and cellular content described in the preceding

10   figures may reasonably be expected to result from the simple binding of Ubc9 to the transporters, the resulting attachment of sentrin residues to the transporters, or both of these mechanisms.

Other embodiments are within the following claims.

What is claimed is:

46.

1.   A method of increasing basal glucose transport, or increasing the level of
the GLUT1 glucose transporter, in a cell or subject comprising:
        administering an antagonist of the cellular protein the cellular protein
Ubc9,
5   thereby increasing basal glucose transport or increasing the level of GLUT1.

2.   The method of claim 1, wherein the increase of basal glucose transport, or
of a level of GLUT1, is unaccompanied by an increase in insulin-stimulated
glucose uptake.
10

3.   The method of claim 1, wherein the increase of basal glucose transport, or
of a level of GLUT1, is unaccompanied by an increase in cell surface GLUT4.

4.   The method of claim 1, wherein the the antagonist of UBC9 is a peptide
15   or protein molecule.

5.   The method of claim 4, wherein the antagonist is an enzymatically
inactive form of Ubc9.

20   6.   The method of claim 4, wherein the antagonist is an enzymatically
inactive form of Ubc9 in which residue 93 of Ubc9 is other than cysteine.

7.   The method of claim 4, wherein the antagonist is an enzymatically
inactive form of Ubc9 in which residue 93 of Ubc9 is alanine.
25

8.   The method of claim1, wherein a subject in need of an increased level of
basal glucose transport is treated.

9.   The method of claim 8, wherein the subject has a disorder characterized
30   by insufficient levels of basal glucose transport, damaged neuronal tissue, tissue
damaged by stroke; hyperglycemia; or an insulin resistant state, is treated.

10.   The method of claim 8, wherein the subject has diabetes.

47.

11   A method of increasing basal glucose transport, or increasing a level of
GLUT1, in a cell or subject comprising:
        inhibiting an interaction between Ubc9 and GLUT1,
thereby increasing basal glucose transport or increasing the level of GLUT1.
5

12.   A method of inhibiting the attachment of sentrin to GLUT1 or GLUT4
comprising:
        inhibiting an interaction between Ubc9 and GLUT1,
thereby inhibiting the attachment of sentrin to GLUT1 or GLUT4.
10

13.   A method of decreasing basal glucose transport, or decreasing a level of
GLUT1, e.g., cell surface GLUT1, in a cell or subject comprising:
        promoting an interaction of Ubc9 or sentrin or a function analog of sentrin
moiety with GLUT4 and/or GLUT1, thereby decreasing the basal glucose
15   transport and/or decreasing the level of GLUT1.

14.   **A mehtod of** decreasing insulin-stimulated glucose transport, or
decreasing a level of GLUT4, in a cell or subject comprising:
        inhibiting an interaction between Ubc9 and GLUT1,
20   thereby decreasing insulin-stimulated glucose transport or decreasing the level of
GLUT4.

15   A method of evaluating a compoundfor the ability to an interaction
between Ubc9 and GLUT4 including:
25   a) providing a GLUT4 moiety;
                b) contacting the GLUT4 moiety with said compound; and
                c) evaluating the ability of the GLUT4 moiety to interact with, e.g., bind,
said compound, the ability of the compound to interact with the GLUT4 moiety
being indicative of the ability to modulate the interaction between GLUT4 and
30   Ubc9, thereby evaluating a compound for the ability to modulate the interaction
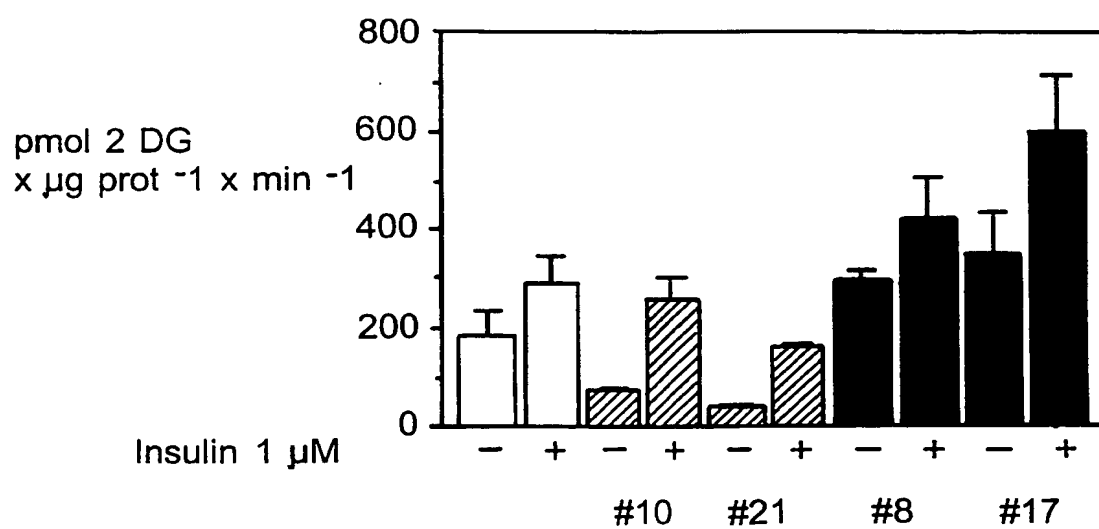between Ubc9 and GLUT4.

48.

16.   A method of evaluating a compound for the ability to modulate an interaction between GLUT4 and Ubc9 comprising:

        a) forming a reaction mixture which includes a Ubc9 moiety, a GLUT4 moiety and the compound; and

5   b) determining if the compound modulates an interaction between the GLUT4 moiety and the Ubc9 moiety.

17.   A fragment of GLUT4 which interacts with Ubc9, and which includes the first eleven amino acids of the C-terminal domain of GLUT4.

10

18   A fragment of GLUT1 which interacts with Ubc9, and which includes the first eleven amino acids of the C-terminal domain of GLUT1.

19.   A fragment of Ubc9 capable of interacting with the first eleven amino

15   acids of the C-terminal domain of GLUT4.

20.   A fragment of Ubc9 capable of interacting with the first eleven amino acids of the C-terminal domain of GLUT1.

20 21.   A GLUT4 or GLUT1 binding fragment of Ubc9 which includes residue 93 of Ubc9.

22.   A method of treating a subject having a disorder characterized by unwanted basal levels (GLUT1-mediated levels) of a glucose transport

25 comprising:

        promoting the attachment of sentrin (or functional analog of sentrin) to GLUT1, thereby decreasing basal glucose transport.

23.   A method of treating a subject having a disorder characterized by

30 unwanted basal levels (GLUT1-mediated levels) of glucose transport comprising:

        promoting the attachment of sentrin to GLUT1, thereby decreasing basal glucose transport.

49.

24.   A method of evaluating a compound comprising:
        a) providing a GLUT4 or 1 moiety;
        b) providing a Ubc9 moiety; and
        c) forming a reaction mixture containing the GLUT4 or 1moiety, the
5   Ubc9 moiety, and other components required for the attachment of sentrin to
GLUT4 or 1 and the
        compound; thereby, evaluating the compound.

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FIG. 1